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PRINCIPAL INVESTIGATOR: **Dr. Brian Ruffell**

CONTRACTING ORGANIZATION:
University of California, San Francisco
San Francisco, CA 94143-0962

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Brian Ruffell, Ph.D.

Email: brian.ruffell@ucsf.edu

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14. ABSTRACT

The presence of some innate immune cell types in developing neoplasms provides a significant pro-tumor advantage. Myeloid-lineage immune cells, such as tumor-associated macrophages (TAMs) and immature myeloid-derived cells/monocytes, promote tumor development by exerting pro-tumor activities including activating angiogenic programs, suppressing anti-tumor immunity, and enhancing migratory and metastatic properties of malignant cells. Cathepsin C (CTSC) is a lysosomal cysteine-class hydrolase expressed in most mammalian tissues. In myeloid cells and cytotoxic lymphocytes, CTSC regulates catalytic activation of several important leukocyte-derived serine proteases including granzymes, chymases and elastases. Preliminary data from the Coussens' laboratory has revealed that in a mouse model of de novo mammary adenocarcinoma development, neither latency of progression to primary tumors, tumor burden, nor tumor histopathology is regulated by CSTC; however, in *cathepsin C (Ctsc)*-deficient mice, there is a significant reduction in the number of circulating malignant cells in peripheral blood, with a corresponding significant reduction in pulmonary metastasis development, thus indicating that CC plays a predominant role in metastatic dissemination of primary mammary tumors. Since CTSC expression in mammary adenocarcinomas is largely localized in TAMs, these provocative data indicate that macrophages play an important role in regulating intravasation and/or survival of malignant cells in peripheral blood, and that this function is at least partly dependent on CTSC.

15. SUBJECT TERMS

breast cancer, mouse models, inflammation, proteases, cathepsin protease, immune cells, leukocytes

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I. INTRODUCTION:

It is now clear that solid tumor development is profoundly regulated by infiltrating immune cells of both the innate and adaptive lineages (1, 2). Diverse populations of leukocytes infiltrate tumor microenvironments and are altered by the neoplastic cells and surrounding stroma in such a way that many of their bioactivities are co-opted to aid growth and/or metastasis of nascent tumors. Out of this realization emerges novel targets for therapy that may impact patient survival, i.e., immune targets regulating primary tumor development, and those regulating metastatic dissemination and growth. Metastasis is a highly complex process and involves multiple stages. First, malignant cells must intravasate into blood vessels and enter the peripheral circulation; second, they must survive in the blood, third, they must extravasate and leave the blood and enter a distal organ; and fourth, they must be able to survive and proliferate in ectopic sites. Preliminary analyses in the Coussens laboratory using a mouse model of breast cancer has revealed that absence of a single intracellular leukocyte lysosomal protease, i.e. cathepsin C (CTSC), reduces the ability of malignant mammary epithelial cells to metastasize to the lungs. As metastasis is a leading cause of mortality in cancer patients, these findings indicate that CTSC is a potential therapeutic target for breast cancer. To examine this possibility, the mechanism by which CTSC regulates pulmonary metastasis will be determined *in vivo*. In addition, we will reveal the therapeutic efficacy of targeting CTSC to inhibit lung metastasis *in vivo*. And finally, we will evaluate a large cohort of human breast caner tissues for CTSC expression, and determine if this molecule can be used as a biomarker to predict clinical outcome and therefore guide therapy.

II. RESEARCH ACCOMPLISHMENTS BODY:

AIM 1: DETERMINE THE FUNCTIONAL ROLE OF CATHEPSIN C IN REGULATING LEUKOCYTE INFILTRATION AND BIOACTIVITY IN MAMMARY ADENOCARCINOMA DEVELOPMENT AND METASTASIS

Establish the profile of cells expressing CTSC during tumor development and metastasis:

Profile CTSC expression in mammary and lung tissue of MMTV-PyMT mice by flow cytometry. (months 1-6)

Despite using bone marrow derived mast cells from *Ctsc*$^{+/-}$ and *Ctsc*$^{-/-}$ mice, we were unable to detect a clear signal by flow cytometry following intracellular staining for CTSC. We therefore optimized staining for CC and other lineage markers for both immune and non-immune populations in PyMT tumors using PFA-fixed frozen tissue. As reported in our initial application, F4/80^{+} macrophages within the tumors were the dominant population expressing CTSC (**Figure 1**), with lower expression of CTSC in other tumor-infiltrating immune cells such as T cells and neutrophils (data not shown). Surprisingly however, the use of CTSC-deficient animals as a staining control revealed low, but consistent, expression of CTSC in other stromal cells such as fibroblasts and pericytes, in addition to the cancer cells themselves. Given the reduced metastatic phenotype in PyMT/*Ctsc*$^{-/-}$ animals, we also stained for CTSC in the lung (**Figure 2**). While a similar expression pattern was observed in immune populations and in metastatic cancer cells, CTSC was not detected in the other stromal populations within the lung.

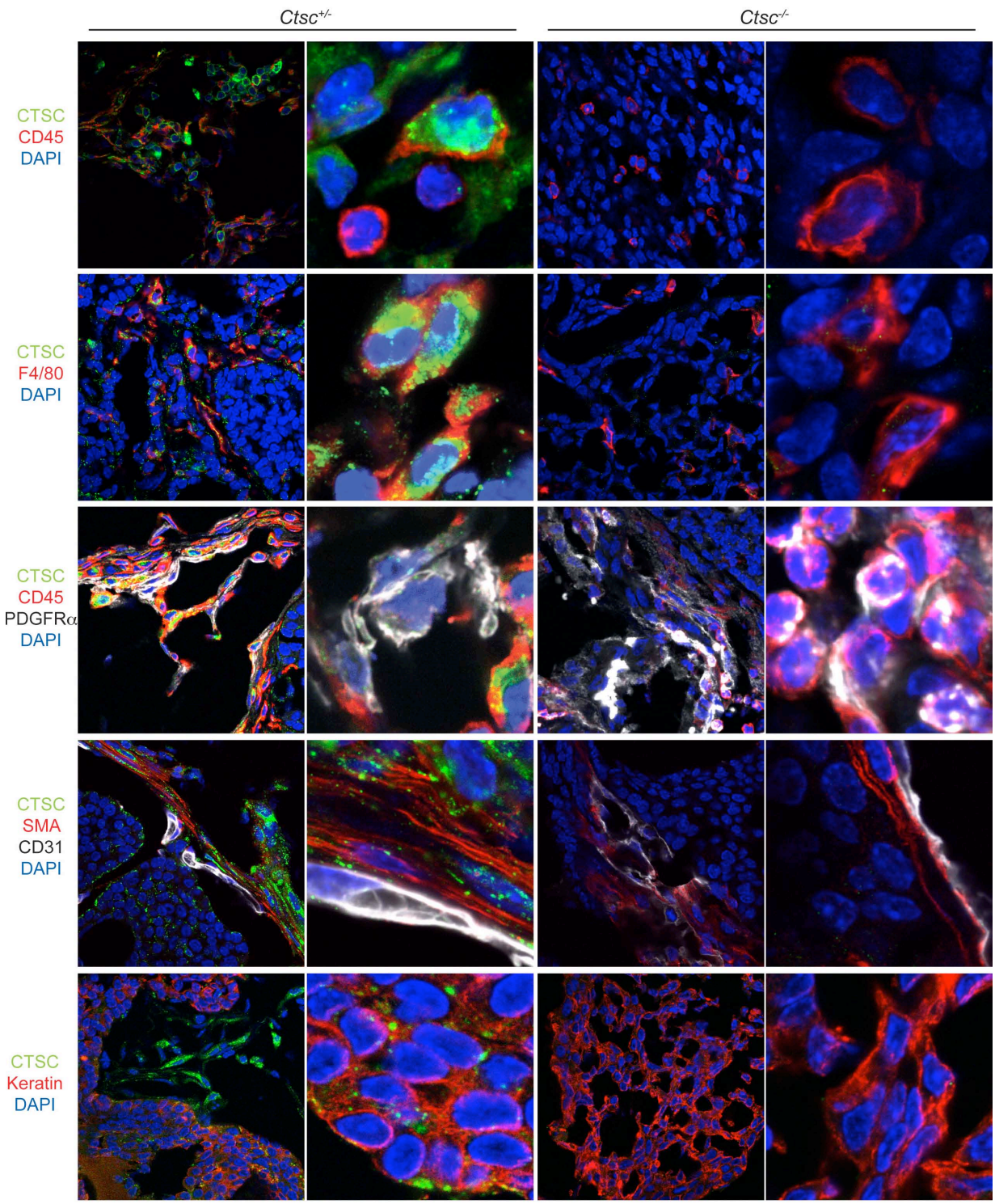

Figure 1: Cathepsin C is primarily expressed by macrophages in primary mammary tumors. Low CTSC expression levels were visualized by confocal microscopy within tumor cells (Keratin$^+$, bottom panels), CD31$^+$ endothelial cells and SMA$^+$ pericytes (2nd from bottom), as well as fibroblasts (PDGFRα$^+$, middle panels). High expression of CTSC was observed in CD45$^+$ leukocytes (top panels) including F4/80$^+$ macrophages (2nd from top).

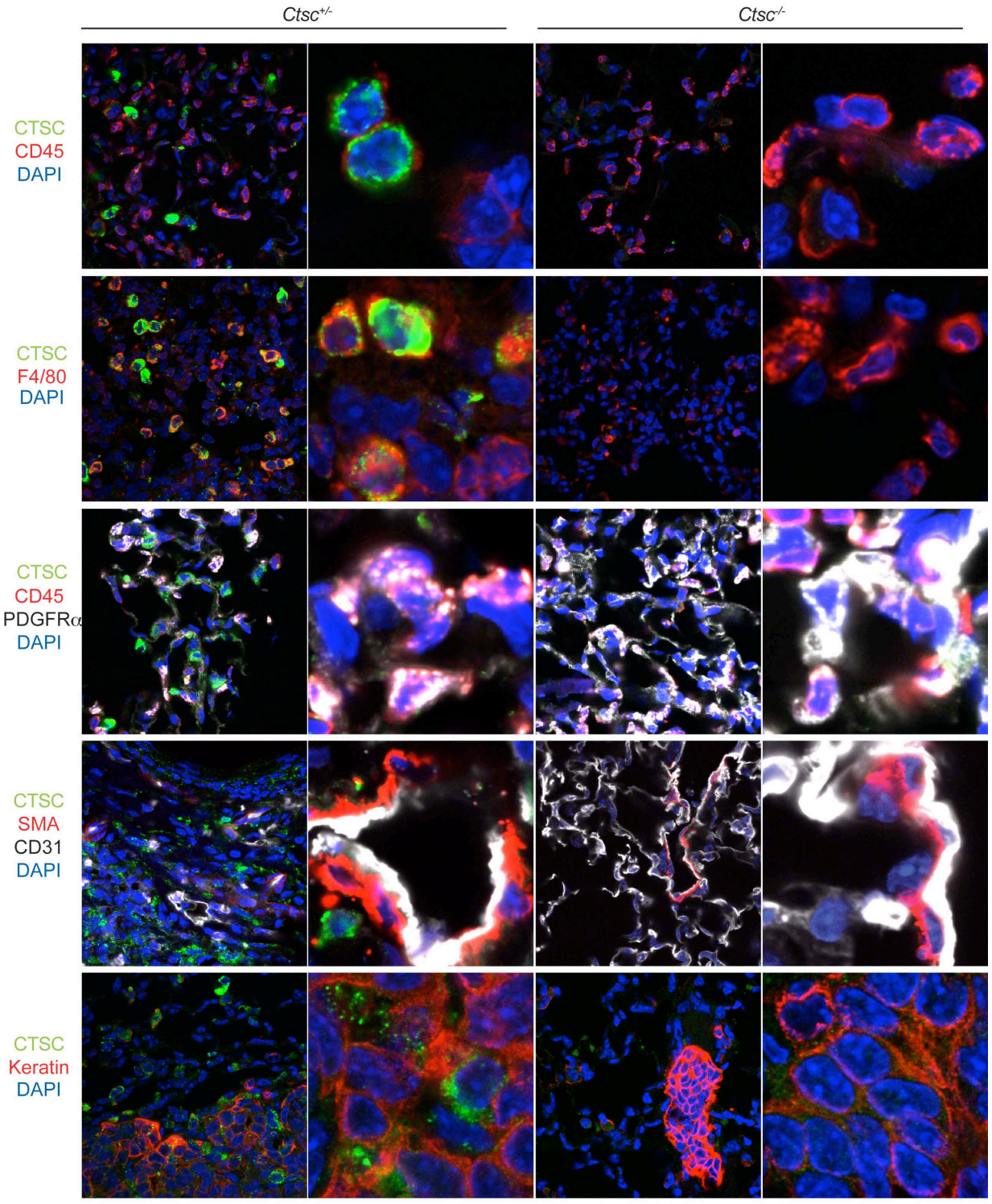

Figure 2: Cathepsin C is primarily expressed by macrophages in metastatic lungs. Low CTSC expression levels were visualized by confocal microscopy within metastatic foci tumor cells (Keratin$^+$, bottom panels), but not in non-leukocytic stromal cells including CD31$^+$ endothelial cells, SMA$^+$ pericytes (2nd from bottom), and fibroblasts (PDGFRα$^+$, middle panels). High expression of CTSC was observed in CD45$^+$ leukocytes (top panels) including F4/80$^+$ macrophages (2nd from top).

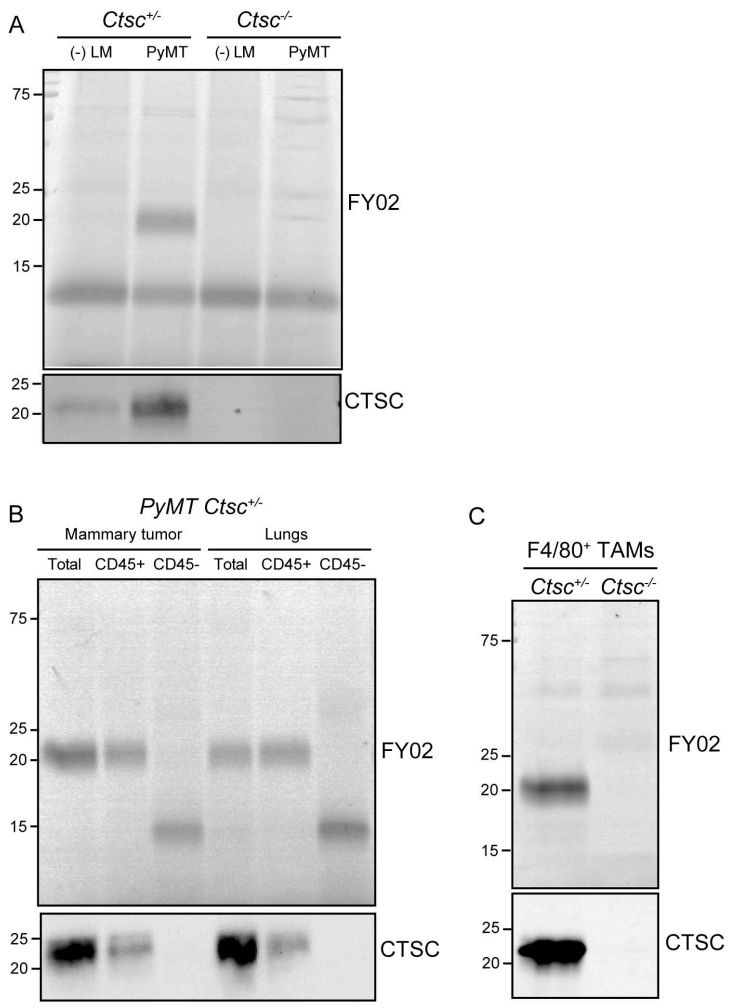

Figure 3: Cathepsin C expression and activity is preferentially found in CD45$^+$ tumor and lungs associated leukocytes. A) FY02 labeling of total cell extracts from mammary glands and carcinomas from *Ctsc*$^{+/-}$ and *Ctsc*$^{-/-}$ PyMT transgenic or negative littermates (-LM). CTSC protein levels were assessed by western blotting for all samples (bottom panel). **B)** FY02 labeling of total cell extracts from non FACS-sorted (total), CD45$^+$ and CD45$^-$ FACS-sorted cells from Ctsc-proficient PyMT mammary carcinomas and lungs. **C)** Same as (B), but for F4/80$^+$ purified TAMs.

Evaluate CTSC activity using a selective probe, FY02, in lysates of purified leukocytes isolated from mammary glands, peripheral blood and lung from MMTV-PyMT mice. (months 3-6)

Detecting CTSC activity in specific leukocyte populations proved technically challenging, as approximately 1x10^6 CD45$^+$ cells were required to either detect a signal with the FY02 probe (*3*), or western blot for CTSC. To demonstrate that CTSC protein expression determined by confocal microscopy corresponded to CTSC activity in leukocytes, CD45$^+$ and CD45$^-$ cells were sorted from PyMT tumors or metastatic lungs and cell lysates were analyzed using the FY02 probe. Both CTSC protein and activity were found only within the CD45$^+$ population (**Figure 3B**), indicating that leukocytes express the majority of active CTSC within both tumors and lungs. Specific purification of F4/80$^+$ macrophages also revealed CTSC activity within this specific leukocyte population (**Figure 3C**). As anticipated from increased gene expression of *Ctsc* in PyMT tumors compared to normal mammary glands (~7-fold, data not shown), higher CTSC activity was also observed (**Figure 3A**).

Quantitatively assess activity of CTSC enzymatic substrates in purified leukocytes using selective protease activity assays (months 6-12)

As discussed below, we have yet to identify the specific leukocyte population responsible for the reduced metastatic phenotype in PyMT/*Ctsc*$^{-/-}$ animals, and even have some preliminary data suggesting that it is expression of CTSC by the tumors cells themselves that is important for the phenotype. Pending identification of the cell type of interest, analysis of CTSC substrate activity is currently on hold.

<u>Assess functional significance of CTSC in regulating macrophage phenotype:</u>

Analyze polarization state of tumor-associated macrophages by intracellular staining for cytokine expression. (months 1-6)

Verify macrophage polarization by iNOS and arginase expression levels by real time PCR. (months 6-9)

Compare the ability of PyMT/*Ctsc*$^{+/-}$ and PyMT/*Ctsc*$^{-/-}$ tumor-associated macrophages to influence invasive properties of malignant MECs grown as organoids using the 3D organotypic model. (months 6-12)

While we originally proposed analyzing macrophage polarization and then examining whether altered polarization resulted in a functional difference in the ability of macrophages to promote invasion, we instead bypassed the early steps to directly determine whether CTSC expression by macrophages was important for this process. As shown in **Figure 4A**, expression of the M2 macrophage marker CD206 and the M1 marker MHCII were unchanged between PyMT/*Ctsc*$^{+/-}$ and PyMT/*Ctsc*$^{-/-}$ macrophages. Furthermore, while as reported the IL-4-dependent polarization of the macrophages promoted the invasive properties of the cancer cells in the 3D organotypic model (*4*), invasion was unaffected by macrophage CTSC expression (**Figure 4B**). Based upon these results, and the preliminary data suggesting intrinsic CTSC expression by cancer cells is critical for lung colonization (see Aim 2), further analysis of macrophage polarization is currently on hold.

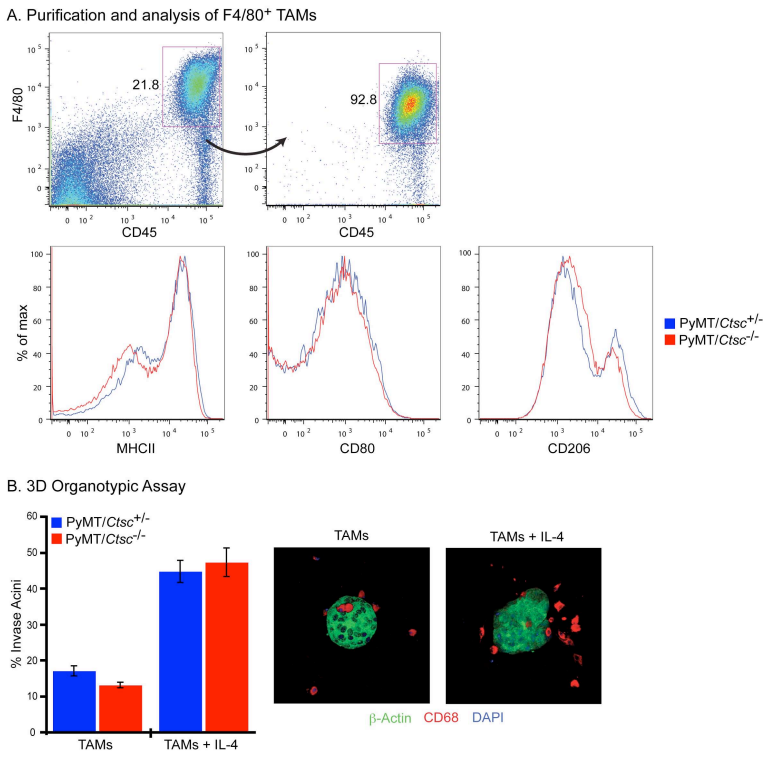

Figure 4: Cathepsin C expression does not influence macrophage-dependent tumor invasion in vitro. A) Purification and analysis of polarization markers. TAMs were purified to >90% via magnetic beads and analyzed for marker expression by flow cytometry. B) 3D organotypic invasion assay with unpolarized or interleukin-4 polarized TAMs from both PyMT/*Ctsc*$^{+/-}$ (blue) and PyMT/*Ctsc*$^{-/-}$ (red) mice. Representative organoids (green) with TAMs (red) are shown on the right, while quantitation of the percent of organoids displaying invasive acini greater than half their diameter is shown on the left.

Evaluate leukocyte infiltration regulated by CTSC in PyMT mice:

Analyze leukocyte infiltration in mammary and lung tumors in PyMT/*Ctsc*$^{+/-}$ and PyMT/*Ctsc*$^{-/-}$ mice using multicolor flow cytometry. (months 1-6)

Measure cytokine expression levels in mammary and lung tumors in PyMT/*Ctsc*$^{+/-}$ and PyMT/*Ctsc*$^{-/-}$ mice via ELISA. (months 3-9)

Identify leukocyte populations with altered cytokine expression profiles in mammary and lung tumors in PyMT/*Ctsc*$^{+/-}$ versus PyMT/*Ctsc*$^{-/-}$ mice using intracellular cytokine staining and multicolor flow cytometry. (months 9-12)

12-color polychromatic flow cytometry was used to analyze tissue invasion by leukocytes and activation/polarization through extracellular markers. As shown in **Figure 5D**, leukocyte populations in both mammary glands and mammary tumors were unchanged between *Ctsc*$^{+/-}$ and *Ctsc*$^{-/-}$ mice, with total leukocyte infiltration was also unchanged (**Figure 5C**). Leukocyte populations within the spleen, lymph node and peripheral blood; and expression of T cell activation markers were also unchanged (data not shown). Based upon our discovery that stromal populations and malignant epithelial cells also expression CTSC (Figure 1), we adapted our flow cytometric protocol to examine cellular composition of tumors. As show in **Figure 5A**, the relative frequency of fibroblasts (PDGFRα^{+}), endothelial cells (CD31^{hi}), pericytes (SMA^{+} or Desmin^{+}) and lymphatic endothelial cells (CD31^{lo}) was unchanged by CTSC expression. Based upon these results, and the preliminary data suggesting intrinsic CTSC expression by cancer cells is critical for lung colonization (see Aim 2), further analysis of cytokine expression by leukocytes is currently on hold.

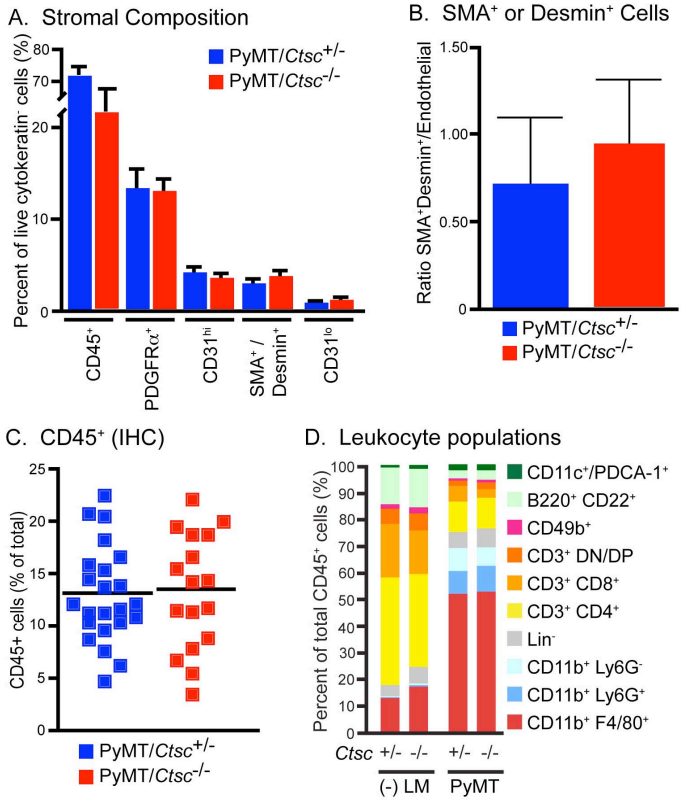

Figure 5: Stromal and leukocytes composition is not altered by the presence of Cathepsin C. A) The stromal composition of mammary tumors determined by polychromatic flow cytometry through analysis of the cytokeratin negative populations in PyMT/*Ctsc*$^{+/-}$ (blue) and PyMT/*Ctsc*$^{-/-}$ (red) animals. **B)** Analysis of A showing the ratio of pericytes (either SMA or Desmin positive) to endothelial cells (CD31^{hi}). **C)** Percent of total cells that are CD45^{+} as determined by immunohistochemistry (IHC). **D)** Composition of leukocytes (CD45^{+} cells) within mammary glands taken from negative littermates (-LM) and mammary tumors from PyMT animals as determined by polychromatic flow cytometry.

AIM 2: DETERMINE AT WHICH STAGE OF CANCER DEVELOPMENT CATHEPSIN C FUNCTIONALLY REGULATES PULMONARY METASTASIS OF MAMMARY CARCINOGENESIS

<u>Determine if cathepsin C regulates intravasation from primary mammary tumors into the circulation:</u>

Measure the number of circulating neoplastic cells in the blood of PyMT/*Ctsc*$^{+/-}$ and PyMT/*Ctsc*$^{-/-}$ mice at day 85 and 95 using either flow cytometry or PCR. (months 12-18)

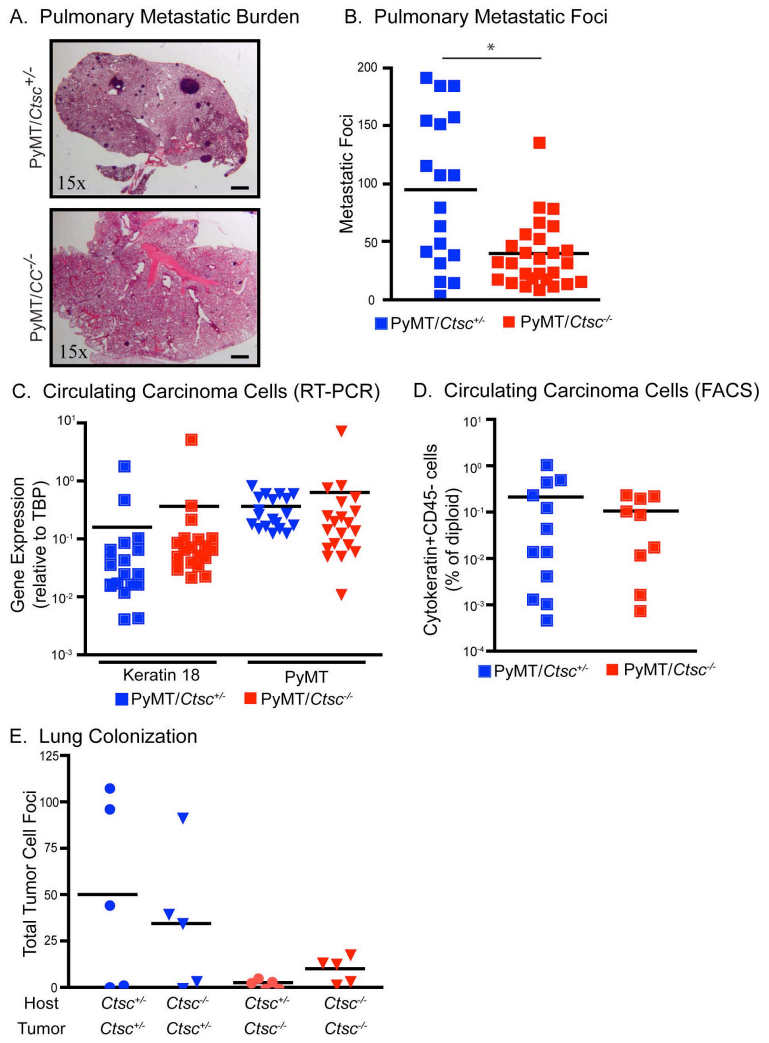

Figure 6: Cathepsin C expression by tumor cells affects lung metastasis but not intravasation from the primary tumor. A) Representative lung sections depicting metastatic tumor burden at day 110 visualized by H&E staining. The scale bar represents 1 mm. **B)** Quantitation of average number of metastatic foci/5.0 mm^2 lung section for PyMT/*Ctsc*$^{+/-}$ (blue) and PyMT/*Ctsc*$^{-/-}$ (red) mice at end stage. **C)** Real-time PCR quantitation of Keratin 18 and PyMT gene expression from circulating cells. Normalization was done with expression of control gene TBP (TATA box binding protein). **D)** Quantitation of circulating cytokeratin$^+$CD45$^-$ cells by flow cytometry. **E)** Lung colonization of *Ctsc*$^{+/-}$ and *Ctsc*$^{-/-}$ negative littermates following injection of carcinoma cells isolated from either PyMT/*Ctsc*$^{+/-}$ or PyMT/*Ctsc*$^{-/-}$ mice.

Preliminary data described in the initial application showed a reduction in the number of circulating carcinoma cells in the blood of PyMT/*Ctsc*$^{-/-}$ mice (n=3). However, increasing the number of animals analyzed has revealed no significant difference in circulating carcinoma cells when measured by flow cytometry (**Figure 6D**), or gene expression of the PyMT transgene or keratin 18 (**Figure 6C**). These results suggest that the ability to extravastate into the blood and survive in circulation are unaffected by CTSC. This supports the data from Figure 4, demonstrating no defect in the ability of CTSC-deficient TAMs to induce invasion.

Examine directional growth of neoplastic cells in the 3D organotypic model during co-culture with the leukocyte populations identified in Aim 1. (months 12-18)

These studies have not yet been initiated.

Examine the ability of leukocyte populations to induce chemotaxis of neoplastic cells using a modified Boyden chamber (months 18-21)

These studies have not yet been initiated.

Adoptive transfer of splenic T cells into PyMT/*Ctsc*$^{-/-}$ mice and evaluation of lung metastasis and the number of circulating MECs. (months 18-24)

These studies have not yet been initiated.

<u>Determine of cathepsin C regulates MEC survival in blood, lung seeding, or outgrowth:</u>
Generate 4 neoplastic cell lines from PyMT/*Ctsc*$^{+/-}$ and PyMT/*Ctsc*$^{-/-}$ mice that are also transgenic for GFP (2 from early non-invasive mammary tumors and 2 from late, invasive tumors). (months 1-6)

Neoplastic cells have been purified from two late stage PyMT/*Ctsc*$^{+/-}$ and PyMT/*Ctsc*$^{-/-}$ mice and one set of cells from each genotype has been used in a preliminary experiment (see below). Breeding issues have delayed generating PyMT/*Ctsc*$^{+/-}$ and PyMT/*Ctsc*$^{-/-}$ mice that also express GFP under control of the β-actin promoter, but these mice have now been generated and mice are aging out.

Intravenously inject neoplastic cell lines in *Ctsc*$^{+/-}$ and *Ctsc*$^{-/-}$ mice and measure MEC survival in the blood, lung infiltration, and outgrowth/tumor progression. (months 12-18)

One experiment has so far been completed wherein neoplastic cells from PyMT/*Ctsc*$^{+/-}$ and PyMT/*Ctsc*$^{-/-}$ mice were intravenously injected into *Ctsc*$^{+/-}$ and *Ctsc*$^{-/-}$ mice and the formation of metastatic lesions analyzed after 14 days. Surprisingly, CTSC-proficient neoplastic cells in 3/5 mice were able to form a substantial number of foci, irrespective of the host genotype (**Figure 6E**). CTSC-deficient neoplastic cells meanwhile, formed few lesions in all mice. Repetition of this experiment is currently underway to determine if this is a valid finding, but if true, it suggests that intrinsic expression of CTSC by tumor cells is important for the later stages of metastasis. Normal levels of circulating carcinoma cells in PyMT animals (Figure 6C and D) suggests this effect would not be due to survival in the blood, but either intravasation into the lung or survival/growth within the lung. GFP expressing neoplastic cells will be used in the future to differentiate between these possibilities.

If extravastion into the lung is affected by cathepsin C expression, then examine rolling, adhesion, and transmigration of neoplastic cell lines on immortalized mouse endothelial cells. (months 18-24)

These studies have not yet been initiated.

If tumor growth or development is altered, inject neoplastic cells subcutaneously in a xenograft model along with purified tumor-associated leukocytes and measure early growth. (months 18-24)

These studies have not yet been initiated.

Determine if metastasis of mammary tumors is regulated by leukocyte-derived CTSC:
Generate bone marrow chimeric mice by using irradiated PyMT/*Ctsc*$^{+/-}$ and PyMT/*Ctsc*$^{-/-}$ mice and the bone marrow from GFP^{+} and GFP^{+}/*Ctsc*$^{-/-}$ mice. (months 12-24)

Analyze tumor incidence, growth, progression and metastasis in the four combinations of chimeric mice. (months 18-36)

If tissue cathepsin C expression appears to be important, generate chimeric mice that do not express PyMT and measure tumor growth in a xenograft model and lung metastasis following intravenous injection. (months 18-36)

These studies have not yet been initiated.

AIM 3: DEVELOP DIAGNOSTIC AND THERAPEUTIC APPROACHES BASED ON CATHEPSIN C BIOACTIVITY TO IMPROVE BREAST CANCER PATIENT SURVIVAL

Develop a probe to measure cathepsin C activity in live mice:
Generate FY01-GdDTPA and FY01-FITC. (months 12-18)

Adjust animal protocol to include use of FY01-GdDTPA and JCP410. (months 12-18)

Evaluate entrance of labeled FY01 into purified TAMs (months 18-21)

Evaluate entrance of labeled FY01 into TAMs in vivo (months 18-21)

Analyze entrance of FY01-GdDTPA into tumors using MRI. (months 21-24)

These studies have not yet been initiated

Evaluate efficacy of a selective cathepsin C inhibitor in reducing pulmonary metastasis:
Examine the effect of the selective cathepsin C inhibitor JCP410 in the 3D organotypic co-culture model. (months 24-30)

Evaluate the ability of JCP410 to inhibit cathepsin C activity in mammary and lung tumors by analyzing post-injection activity by MRI or staining unfixed tissue sections. (months 24-36)

Evaluate the efficacy of JCP410 in inhibiting pulmonary metastasis through bi-weekly i.p injection in mice of age 60 and 80 days. (months 24-36)

These studies have not yet been initiated

Evaluate cathepsin C activity during human breast cancer progression:
Submit proposal for use of human tissue (months 12-24)

Analyze expression of cathepsin C in fixed sections of human mammary tumors by double staining for cathepsin C and leukocyte markers followed by confocal microscopy. (months 24-36)

If cathepsin C expression is observed, measure activity in unfixed tissue sections using FY02 (months 24-36)

Correlate cathepsin C expression and activity with information available about the clinical stage of the cancer and/or the outcome. (months 30-36)

Analyze breast cancer tissue microarray data with 10 year follow up data for a correlation between cathpesin C expression and clinical stage and/or outcome. (months 30-36)

Evaluate role of human leukocytes to promote invasive growth in the 3D organotypic co-culture model using human breast cancer cell lines (months 24-30)

Evaluate ability of JCP410 to inhibit the promotion of invasive growth by leukocytes (months 30-36)

These studies have not yet been initiated

III. KEY RESEARCH ACCOMPLISHMENTS:

Aim 1: Determine the functional role of cathepsin C in regulating leukocyte infiltration and bioactivity in mammary adenocarcinoma development and metastasis

Months 1-12

- Predominant expression of CTSC by TAMs in mammary tumors and lungs.
- Low expression levels of CTSC by stromal and neoplastic cells in tumors.
- CTSC activity and protein levels increased in tumors versus mammary glands.
- CTSC activity localized to leukocytes and TAMs in mammary tumors.
- CTSC expression by TAMs does not affect their ability to induce invasion by MECs.
- CTSC expression does not affect the composition of leukocytes within the tumor.
- CTSC expression does not affect the composition of the stromal compartment.

Aim 2: Determine at which stage of cancer development cathepsin C functionally regulates pulmonary metastasis of mammary carcinogenesis

Months 1-12

- CTSC expression does not influence the number of circulating neoplastic cells
- Preliminary data suggesting CTSC expression by neoplastic cells is important in mediating the later stages of lung metastasis.

Aim 3: Develop diagnostic and therapeutic approaches based on cathepsin C bioactivity to improve breast cancer patient survival

Months 1-12

- These studies have not yet been initiated

IV. REPORTABLE OUTCOMES:

Manuscripts:

Ruffell, B., D. G. Denardo, N. I. Affara, and L. M. Coussens. 2010. Lymphocytes in cancer development Polarization towards pro-tumor immunity. *Cytokine Growth Factor Rev* 21:3-10.

Abstracts/Posters:

- *'Role of Cathepsin C During Breast Cancer Metastasis'*, Breast Oncology Program Retreat. San Francisco, CA. January 29-30, 2009.
- *'Role of Cathepsin C During Breast Cancer Metastasis'*, Diller Building Scientific Symposium. San Francisco, CA. August 27-28, 2009.

Presentations:

- '*Role of Cathepsin C During Breast Cancer Metastasis'*, Stanford Univ. School of Medicine. August, 2009.
- '*Role of Cathepsin C in Cancer Progression'*, Pacific Coast Protease Meeting, Borrego Springs, CA. April 2010.

V. CONCLUSION:

It is now clear that infiltrating immune cells profoundly regulate solid tumor development. Diverse populations of cells infiltrate tumor microenvironments and are altered by cancer cells and surrounding stroma in such a way that many of their bioactivities are co-opted to aid growth and/or metastasis of tumors. Out of this realization emerges novel targets for therapy that may impact patient survival, i.e., immune targets regulating primary tumor development, and those regulating metastatic dissemination and growth. It is thus hypothesized that manipulating the immune response and neutralizing its effect on neoplastic cells represents an efficacious alternative approach to current disease management. Preliminary analyses using a mouse model of breast cancer supports this hypothesis, by demonstrating that absence of a single immune cell enzyme reduces the ability of malignant breast cancer cells to metastasize to the lungs. Results from this study will therefore prove significant for elucidation of an important regulatory cascade affecting pulmonary metastasis. Furthermore, as metastasis is a leading cause of mortality in breast cancer, this research may lead to the identification of a novel therapeutic target for reducing breast cancer mortality. This is an innovative approached to formulating a novel anti-cancer therapy, as it targets a molecule expressed by normal cells. This contrasts with the traditional development of anti-cancer therapies, which are most often based upon molecules expressed by cancer cells that are important for their survival or growth. This innovative approach has two potential advantages over the traditional method: it avoids selecting for treatment-resistant neoplastic cells by instead targeting the pro-tumor activities of the microenvironment; and by targeting a specific pro-tumor pathway, there is a strong possibility of developing a treatment with low toxicity.

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4. DeNardo, D. G., J. B. Baretto, P. Andreu, L. Vasquez, N. Kolhatkar, D. Tawfik, and L. M. Coussens. 2009. CD4+ T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *Cancer Cell* 16:91-102.

VII. APPENDICES:

A. Complete academic Curriculum vitae for Dr. Brian Ruffell
B. Collected publications from months 1-12.

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Brian Howard Ruffell	Postdoctoral Scholar University of California, San Francisco

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of British Columbia, Vancouver, BC	BSc	2001	Cell Biology/Genetics
University of British Columbia, Vancouver, BC	PhD	2008	Immunology
University of California, San Francisco, CA	n/a	Current	Cancer Immunology

POSITIONS AND HONORS:

1997 - 2001 Undergraduate Student: Department of Science. University of British Columbia, Vancouver, BC, Canada

2002 - 2008 Graduate Student: Department of Microbiology and Immunology. University of British Columbia, Vancouver, BC, Canada

2008 – pres. Postdoctoral Scholar: Department of Pathology, University of California, San Francisco, San Francisco, CA.

FELLOWSHIPS AND AWARDS:

2009 DOD Breast Cancer Research Program Postdoctoral Award
2006 Heart and Stroke Foundation of Canada Doctoral Research Award
2006 Robert Emmanuel and Mary Day Endowment Award
2005 John Richard Turner Fellowship in Microbiology
2005 Armauer-Hansen Memorial Scholarship
1998 Outstanding Student Initiative Scholarship
1997 British Columbia Government Scholarship
1997 Canadian Armed Forces Memorial Scholarship
1997 Outstanding Student Initiative Scholarship

PEER REVIEWED PUBLICATIONS:

1. **Ruffell B,** DeNardo DG, Affara, NI, Coussens LM. (2010) Lymphocytes in cancer development: polarization towards pro-tumor immunity. Cytokine Growth Factor Rev. 21(1): 3-10.
2. Johnson P, **Ruffell B**. (2009) CD44 and its role in inflammation and inflammatory diseases. Inflamm. Allergy Drug Targets. 8(3): 208-220.
3. **Ruffell B**, Johnson P. (2009) The regulation and function of hyaluronan binding by CD44 in the immune system. Invited review for "Glycoforum" www.glycoforum.gr.jp
4. **Ruffell B**, Johnson P. (2008) Hyaluronan induces cell death in activated T cells through CD44. J. Immunol. 181: 7044-7054.
5. **Ruffell B**, Johnson P. (2005) Chondroitin sulfate addition to CD44H negatively regulates hyaluronan binding. Biochem. Biophys. Res. Commun. 334(2): 306-312.
6. Khan AI, Kerfoot SM, Heit B, Liu L, Andonegui G, **Ruffell B**, Johnson P, Kubes P. (2004) Role of CD44 and hyaluronan in neutrophil recruitment. J. Immunol. 173(12): 7594-7601.

MANUSCRIPTS IN PREPARATION:

7. **Ruffell B**, Brown KL, Tjew SL, Johnson P. Chondroitin sulfate addition to CD44 regulates hyaluronan binding in macrophages.

PUBLISHED ABSTRACTS:

1. **Ruffell B**, Johnson P. (2004) Chondroitin sulfate chains on CD44 negatively regulate its ability to bind hyaluronan. Glycobiology 14(11): 1108.
2. **Ruffell B**, Johnson P. (2006) Hyaluronan induces apoptosis through CD44 in activated lymphoma cells. EJC Supplements 4(12): 147.

POSTER PRESENTATIONS:

1. US/Japan Glycobiology 2004. Honolulu, HI. November 17-20, 2004
2. 18th EORTC-NCI-AACR: Symposium on Molecular Targets and Cancer Therapeutics. Prague, Czech Republic. November 7-10, 2006
3. Breast Oncology Program Retreat. San Francisco, CA. January 29-30, 2009. **Winner of "Best-in-Show Poster Presentation" Award**
4. Diller Building Scientific Symposium. San Francisco, CA. August 27-28, 2009.

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1. US/Japan Glycobiology 2004. Honolulu, HI. November 17-20, 2004
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3. Breast Oncology Program Retreat. San Francisco, CA. January 29-30, 2009.
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6. Molecular and Cellular Biology of Immune Escape in Cancer. Keystone, CO. February 7-12, 2010

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1. Life Sciences Institute, Vancouver, BC (Jan, 2008)
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5. Susan Love Research Foundation, Los Angeles, CA (Jan, 2009)
6. Stanford University School of Medicine, Stanford, CA (Aug, 2009)
7. Pacific Coast Protease Meeting, Borrego Springs, CA (April, 2010)

ADDITIONAL TRAINING:

1. BD LSRII Operator Training Course (August, 2009)

TEACHING:

2003 - 2006 Teaching Assistant, Department of Microbiology and Immunology, University of British Columbia, Vancouver, BC, Canada

MENTORING:

Dates	Name	Program	Role	Current Position
2003	Patrick Yang	Summer Student	Supervisor	PhD/MD Program
2006-2008	Grace Poon	Co-op / Graduate Student	Mentor	PhD Program
2010	Lucia Cottone	PhD Exchange Student	Supervisor	PhD Program
2010	Heather Chan	Undergrad Summer Student	Supervisor	Undergraduate

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Lymphocytes in cancer development: Polarization towards pro-tumor immunity

Brian Ruffell [a], David G. DeNardo [a], Nesrine I. Affara [a], Lisa M. Coussens [a,b,*]

[a] Department of Pathology, University of California, San Francisco, CA 94143, United States
[b] Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, CA 94143, United States

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ABSTRACT

The classic view that the role of immune cells in cancer is primarily one of tumor rejection has been supplanted by a more complex view of leukocytes having both pro- and anti-tumor properties. This shift is due to the now well recognized capabilities of several myeloid cell types that foster pro-tumor programming of premalignant tissue, as well as the discovery that subsets of leukocytes also suppress development and effector functions of lymphocytes important for mediating anti-tumor immunity. In this review, we focus on the underappreciated role that T lymphocytes play in promoting tumor development. This includes, in addition to the role of T regulatory cells, a role for natural killer T cells and $CD4^+$ T helper cells in suppressing anti-tumor immunity and promoting cancer growth and metastasis.

1. Introduction

Leukocyte infiltration into developing *tumors* is now considered one of the hallmarks of cancer development [1]. It is thought that the initial immune response to an early neoplasm mirrors the response to acute tissue injury, with sequential infiltration by various myeloid populations leading to eventual infiltration by lymphocytes [2]. However, as the kinetics of tumor development and the neoplastic cells themselves alter the local immune microenvironment, making inferences between an immune response to injury/infection and tumor development is difficult. Regardless, if clearance of the would-be cancer cells is not achieved and the initial acute inflammatory response fails to resolve, there inevitably results a state of chronic inflammation within the local tissue. It is now well established that chronic inflammation fosters early cancer development through a number of mechanisms mediated primarily by myeloid-lineage cells, including tumor-associated macrophages, immature myeloid cells that can possess suppressive activity, and Tie2-expressing monocytes [3,4]. The immune microenvironment of a neoplastic tissue encompasses not only the composition of infiltrating leukocytes, but also the bioeffector function of these cells within the tissue. Thus, both the presence of a cell within a tumor and expression of tissue-specific cytokines, chemokines and other immune mediators profoundly influence whether an anti-tumor or pro-tumor immune response is elicited [4,5].

Although responding to tissue damage in the form of inflammatory cues, tumor-infiltrating myeloid cells rapidly respond to soluble and insoluble signals emanating from the neoplastic microenvironment. Responses take the form of dramatically altered gene expression programs that alter bioeffector functions of the immune cells. These often result in increased expression of factors/mediators that enhance growth and survival of neoplastic cells, as well as activating and sustaining angiogenic responses, furthering tissue remodeling, and squelching anti-tumor immune programs [4]. Chronic inflammation in tissue resulting from infection or autoimmune disease can also alter the risk of cancer development by providing an environment permissive for initiated preneoplastic cell survival and subsequent proliferation, as well as through production of DNA damaging compounds such as reactive oxygen and nitrogen species that increase mutation frequency [6]. While all of these aspects of solid tumor development are susceptible to regulation by infiltrating immune cells, in the context of this review, we will focus on aspects of carcinogenesis regulated by infiltrating lymphocytes, as mechanisms regulated by myeloid cells have been reviewed elsewhere [5–9].

1.1. T lymphocytes

T cells develop in the thymus from a common lymphoid progenitor and are defined by expression of a T cell receptor (TCR) that is responsible for recognizing antigens presented by the major histocompatibility complex (MHC) family of genes (also called human leukocyte antigen or HLA). T cells are classically divided into either $CD8^+$ cytotoxic lymphocytes (CTL) or $CD4^+$ T helper (T_H) cells that recognize peptides presented by MHCI or MHCII, respectively (Fig. 1). T_H cells are further divided into interferon

* Corresponding author at: Department of Pathology, 513 Parnassus Avenue, HSW-450C, University of California, San Francisco, CA 94143, United States.
E-mail address: lisa.coussens@ucsf.edu (L.M. Coussens).

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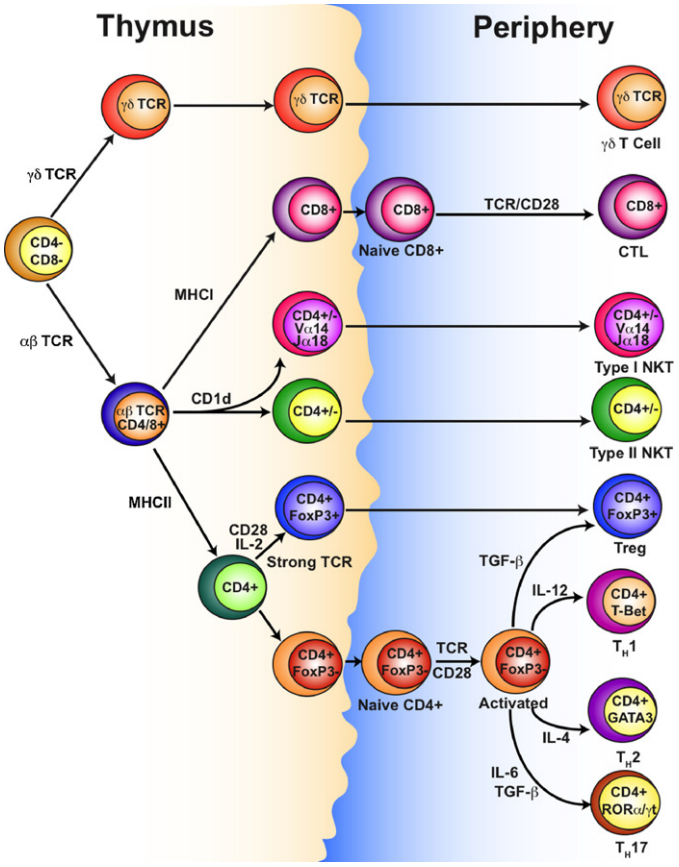

Fig. 1. T cell lineages and subsets. Successful rearrangement and expression of a TCR determines lineage commitment between γδ and αβ T cells. Recognition by αβ T cells of MHCI, MHCII, or CD1d drives $CD8^+$, $CD4^+$ or NKT cell development, respectively. Strong recognition of peptide:MHCII complexes by $CD4^+$ cells drives natural T_{Reg} cell development in the thymus, otherwise $CD4^+$ T cells differentiate into T_H1, T_H2, T_H17 or inducible T_{Reg} cells following activation in the periphery, with polarization directed by IL-4, IL-6, IL-12 and TGF-β. Type I NKT cells are defined by expression of specific α-chain regions (Vα14-Jα18 in mice, Vα24-Jα18 in humans), but the reason for functional differences between type I and type II NKT cells is unclear.

(IFN)-γ and tumor necrosis factor (TNF)-α expressing T_H1 cells and interleukin (IL)-4, IL-5 and IL-13 expressing T_H2 cells. This simplified view of the T cell compartment has been expanded upon by the identification of a range of additional subtypes, including T follicular helper cells (T_{FH}), IL-17 expressing T_H cells (T_H17), and regulatory T cells (T_{reg}) [10]. Paralleling these subtypes in the $CD4^+$ T cell compartment, type 1, type 2, and type 17 $CD8^+$ T cells (T_C1, T_C2, T_C17), as well as regulatory $CD8^+$ cells, have all been described [11–13]. There also exist two 'innate-like' T cell subsets that can be activated either by cytokines or TCR stimulation. Natural killer T (NKT) cells recognize glycolipids presented by the non-classical MHC molecule CD1d [14], while γδ T cells are not MHC restricted and recognize a diverse range of molecules, including soluble non-protein antigens [15]. All of these T lymphocyte subsets have been examined for their role in tumor development and anti-tumor immunity, each with unique roles in directing the immune response.

1.2. Cytotoxic T lymphocytes

Mice harboring specific immune-based genetic deficiencies are more susceptible to formation of carcinogen-induced sarcomas, and depending on the specific defect, are also more prone to develop certain spontaneous tumors and lymphomas [16,17]. The ability of immune-deficient mice to reject and/or inhibit the growth of many, but not all cell lines is also impaired. Numerous studies have shown that, due to their ability to produce IFNγ and directly kill target cells, both $CD8^+$ CTLs and natural killer (NK) cells are the critical mediators of the anti-tumor response [16]. γδ T cells, which share characteristics with both CTLs and NK cells, are also involved in the anti-tumor response in epithelial tissues such as the skin [18], where they can be the dominant T cell population [15]. The relative importance of CTLs, NK cells, and γδ T cells is highly dependent upon the cancer model being used. Even in the skin, genetic deficiency of αβ T cells increases sarcoma formation following administration of methylcholanthrene (MCA), but not 7,12-dimethybenz[a]anthracene (DMBA) or 12-O-tetradecanoyl-phorbol-13-acetate (TPA) [18], while the absence of $CD8^+$ T cells does not influence the development of neoplasms in a mouse model of de novo squamous carcinogenesis, e.g., K14-HPV16 mice [19].

Epidemiological studies of cancer incidence in acquired immune deficiency syndrome (AIDS) and organ transplant patients reveal that the relative risk (RR) for cancer development varies considerably depending upon organ site and cancer etiology [17,20] where viral-associated cancers, in particular Human Herpes Virus 8-associated Kaposi's sarcoma, Epstein-Barr virus-associated Non-Hodgkin's lymphoma and HPV-associated squamous carcinoma, are elevated in immune suppressed individuals due largely to lack of protection against viral infections or viral re-activation in the absence of T cells [21]. That said, some cancer types occur with increased frequency in selected groups of immune compromised patients for reasons unrelated to infection for example, chronic exposure to carcinogens (tobacco) for thoracic malignancies, whereas head and neck, esophageal, gastrointestinal and pancreatic cancers are increased in liver transplant patients associated with prior history of alcohol (and tobacco) use [22,23]. On the other hand, the RR for the most common non-viral-associated solid tumors of epithelial origin, including breast and prostate, are decreased in immune suppressed patients, with some of these having a RR less than 1.0 [17,20].

Examination of T cell infiltrates in tumors reveals cells that can display activation markers and are able to recognize tumor antigens [16], indicating that some tumors are indeed immunogenic and can induce an anti-tumor immune response. Tumor antigens encompass both neo and overexpressed antigens, e.g., c-myc, HER-2/neu and p53 [24], as well as host/stromal cell-derived antigens unique to individual tumors. Clinical studies have even reported accumulation of autoantibodies against extracellular matrix (ECM) components, including anti-collagen type I, III and V, as well as anti-fibronectin antibodies that accumulate in lung cancer and nasopharyngeal patients [25]. How then do neoplastic cells, expressing mutant proteins in an *inflammatory* microenvironment that seemingly engenders a robust T cell response, avoid killing by cytotoxic cells? Importantly, lymphocytes do not act in isolation, and their effector functions are largely dependent upon the release of cytokines and binding of inhibitory and activating receptors to ligands expressed by other leukocytes, stromal cells, and even neoplastic cells. For example, NK cells are potent regulators of $CD8^+$ T cell responses through their release of IFNγ, which provides a maturation signal for tissue resident DCs and assists in $CD8^+$ T cell effector function; meanwhile, cytokines released by mature DCs and activated T cells are important for promoting NK cell effector function [26]. These interweaving regulatory pathways are necessary to initiate, direct, maintain and eventually shutdown an appropriate immune response. Such pathways are also necessary to prevent an inappropriate immune response: despite central tolerance through negative selection of self-reactive lymphocytes, peripheral tolerance mediated by cytokines, inhibitory receptors and immune regulatory cell types is necessary to prevent autoimmune disorders. As cancer cells are

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largely recognized as 'self', it is not surprising they utilize similar mechanisms that effectively dampen anti-tumor immunity; thus, T lymphocytes are intimately involved in regulating both pro- and anti-tumor immunity and chronic inflammation within the tumor microenvironment.

1.3. Regulatory T cells

Since the rediscovery of suppressor T cells as $CD4^+CD25^+$ regulatory T cells, and their further characterization as cells expressing glucocorticoid-induced tumor necrosis factor receptor (GITR), cytotoxic T lymphocyte antigen (CTLA)-4, and uniquely, the transcription factor forkhead box P3 (FoxP3), this T cell subset has become an intense focus of cancer research. T_{Reg} cells can develop in the thymus or can be converted in the periphery by exposure to transforming growth factor (TGF)-β [27]. These 'natural' and 'inducible' T_{Reg} cells, respectively, utilize the same mechanisms to mediate immune suppression and may perform overlapping functions [28]. A number of regulatory T cells that do not conform to the $CD4^+CD25^+FoxP3^+$ phenotype have also been described, but these remain poorly characterized [29] and evidence of a role for these cells in cancer has so far been limited to the isolation of IL-10 producing $CD8^+$ T cells from human ovarian tumors [30]. Immune suppression of $CD4^+$ and $CD8^+$ T cell responses by T_{Reg} cells is mediated both in the secondary lymphoid organs where T cell activation occurs, and in the tissues [31]. Interestingly, in a pancreatic islet allograft model, T_{Reg} cells were shown to enter the inflamed tissue and then migrate to the lymph nodes, where these sequential series of steps was found to be associated with increasing graft survival time [32].

While a host of molecules have been described as important for mediating T_{Reg} cell suppression, these molecules have been broadly classified as acting in one of four ways [33]: (*i*) cytokine inhibition, such as with TGF-β, IL-35 and IL-10; (*ii*) direct cytolysis of effector T cells through perforin and granzyme; (*iii*) metabolic disruption, such as IL-2 deprivation and cyclic adenosine monophosphate (cAMP) transfer; and (*iv*) inhibition of DC function, such as through binding of CTLA-4 to CD80/86 and the induction of indoleamine 2,3-dioxygenase (IDO). These mechanisms appear to have overlapping but non-redundant roles, with the degree of importance being tissue and model dependent [34]. This may be significant in cancer, as $CD4^+CD25^{high}$ tumor-infiltrating T lymphocytes from patients with head and neck squamous cell carcinoma (HNSCC) were found to mediate suppression through IL-10 and TGF-β, while the same population from peripheral blood did not express these cytokines and were less able to suppress proliferation [35]. A role for IL-10 and TGF-β has also been revealed in mice following injection of a fibrosarcoma cell line [36], while a role for perforin- and granzyme B-dependent killing of NK and $CD8^+$ T cells was found in mice after injection of a lymphoma cell line [37]. Other than these studies, the mechanisms by which T_{Reg} cells mediate suppression in cancer have been largely ignored. Instead, research has been focused on correlating T_{Reg} cell infiltration with prognosis and attempts to deplete T_{Reg} cells with anti-CD25 antibody based therapies [29].

Suppression by T cells in solid tumors was first suggested by Fujimoto et al. [38], cumulating in work by North and colleagues, who showed that the ability to reject a second subcutaneous injection of a fibrosarcoma cell line was inversely correlated to increased suppressor activity of $CD3^+CD4^+$ cells over time [39,40]. These findings have since been expanded to show that depletion of $CD25^+$ cells, which are largely $CD4^+FoxP3^+$, reduces tumor growth of some tumor cell lines [29], as well as MCA-induced fibrosarcomas [41,42]. Infiltration of T_{Reg} cells into the tumor is observed in all of these models, while an increased percentage of T_{Reg} cells in the periphery is observed only in some instances. In one study using a fibrosarcoma cell line, depletion of $CD4^+$ cells resulted in $CD8^+$ T cell-dependent tumor regression in 50% of the mice [36], that was increased to 100% following either $CD4^+$ or $CD25^+$ cell depletion when another fibrosarcoma-derived cell line expressing a strong antigen was used [36]. Together with other studies, experimental systems such as these indicate that initiation of a $CD8^+$ T cell response is possible during tumor development, depending on the immunogenicity of the tumor antigens involved, but that local and/or systemic immune suppression by T_{Reg} cells can limit their effectiveness. Unfortunately, these results have been limited in scope to transplantable tumor models representing few tumor types, and thus further investigation is required to determine whether T_{Reg}-dependent immune suppression is applicable to a wide range of spontaneous tumors, as might be expected.

Increased number of tumor-infiltrating $FoxP3^+$ cells is associated with poor prognosis in several cancers, including hepatocellular carcinoma [43], ovarian carcinoma [41], pancreatic ductal carcinoma [44], cervical cancer [45], non-small cell lung carcinoma [46], HNSCC [35], and breast cancer [47,48]; with varying degrees of prognostic value regarding patient outcome. As with some mouse models, increased frequency of T_{Reg} cells in peripheral blood of some cancer patients has been reported [49]. Peripheral blood T_{Reg} cells from patients with ovarian cancer displayed equal suppressive capacity compared to tumor-derived T_{Reg} cells [41]. This contrasts with the study of patients with HNSCC [35], highlighting the potential for differences in the immune response based on cancer type and/or etiology.

Attempts to translate T_{Reg} research into the clinic have focused around $CD25^+$ cell depletion using denileukin diftitox. Known commercially as Ontak, this compound composed of IL-2 fused to a portion of diphtheria toxin has been approved for treating $CD25^+$ cutaneous T cell leukemia and lymphoma [29]. Ontak administration has been demonstrated to reduce the numbers of peripheral T_{Reg} cells and improve T cell activation in a small number of patients with either lung, ovarian, breast, or renal cancer; either alone or in combination with DC-based vaccination [50,51]. Other small studies have confirmed these findings, but as before, objective clinical responses were rare. Furthermore, a larger study with NSCLC patients observed no objective clinical responses, and almost half of the patients suffered side effects usually associated with IL-2 immunotherapy [52]. Dosage optimization thus remains an issue, as does route of administration, as intratumoral injection of anti-CD25 antibodies demonstrated efficacy in mouse models [36]. Determination of which cancer types are most suitable for T_{Reg} depletion is also required, as the ability of $CD25^+$ cell depletion to improve anti-tumor immunity is known to depend on the tumor type [53]. Finally, combinatorial therapies, such as $CD25^+$ cell depletion with blockage of immunosuppressive molecules such as CTLA-4 and programmed death (PD)-1 [54], have great potential to overcome immune suppression within the tumor, although the induction of autoimmune diseases will likely remain an issue for patients.

1.4. T helper cells

Lineage commitment between $CD4^+$ and $CD8^+$ T lymphocytes occurs during development within the thymus. Further differentiation of the $CD4^+$ lineage, with the exception of natural T_{Reg} cell development, requires T cell activation through MHCII and co-stimulatory molecules, as well as cytokine dependent signaling that is responsible for directing the cell towards a particular lineage (Fig. 1). Classical differentiation of $CD4^+$ T cells into T_H1 and T_H2 cells, mediated by IL-12 and IL-4 respectively, was recently updated to include a new T_H17 lineage [10,55]. T_H17 cells are induced by a combination of IL-6 and TGF-β and mediate their

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effects through secretion of IL-17(A), IL-17F, IL-21 and IL-22 [56]. Many of these cytokines increase the severity of autoimmune diseases in mouse models by promoting inflammation, but also appear to be functionally important in protecting against some extracellular pathogens, possibly by mediating leukocyte recruitment through the induction of chemokine expression [57,58].

T_H17 cells have been observed in patients with ovarian [59], prostate [60] and gastric cancer [61], and high numbers of IL-17 producing cells in hepatocellular carcinoma patients is an indicator of poor prognosis [62]. In mice, transgenic expression of IL-17 by cell lines increases tumor growth by promoting angiogenesis [63,64], knockdown of the IL-17 receptor in 4T1 mammary carcinoma cells reduces survival and tumor growth [65], and IL-17 depletion delays development of chemically induced papillomas [66]. This effect appears to be mediated by a pathway of IL-17 inducing IL-6 production by both neoplastic and stromal cells, that in turn leads to activation of Stat3 [67]. Not only is Stat3 involved in upregulating genes that promote tumor growth and immune suppression [68,69], but it also mediates expression of IL-17 [70], potentially leading to a dangerous feedback loop (Fig. 2).

However, as with inflammation in general, IL-17-dependent inflammation may have both positive and negative effects on tumor growth, depending on the tumor model. MC38 colon cancer cell growth is enhanced in IL-17-deficient mice [71], while adoptive transfer of *in vitro* polarized T_H17 cells specific for a B16 melanoma antigen can induce tumor regression [72]. This effect appears to depend upon IFNγ, as blocking antibodies prevent T_H17 cell transfer from causing tumor regression [72], while reduced frequency of IFNγ producing cells were observed in IL-17-deficient tumors [71]. Adding to the confusion is that both IL-21 and IL-22 activate Stat3, and IL-21 can promote Th17 differentiation [56]. Meanwhile, $CD8^+$ T cells primed *in vitro* in the presence of IL-21 provide a more robust anti-tumor response upon adoptive transfer, and IL-21 therapy is currently in clinical trials for cancer treatment [73].

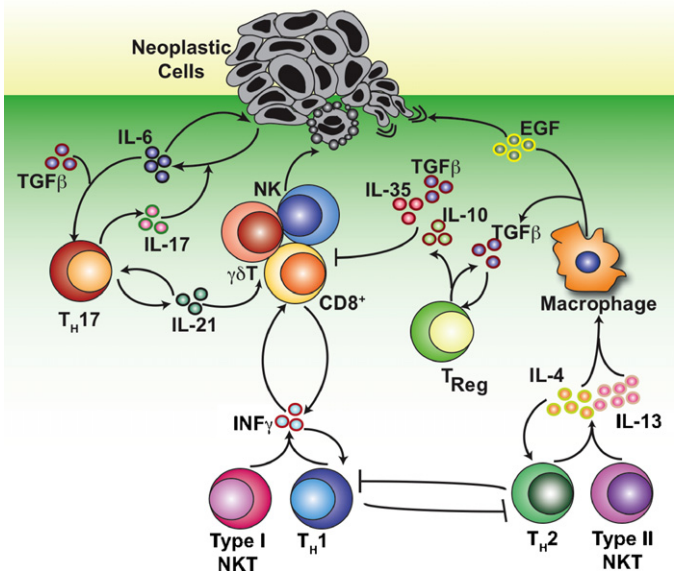

Fig. 2. T cell-derived cytokines regulate pro- and anti-tumor immunity. NK cells, γδ T cells, and $CD8^+$ CTLs mediate anti-tumor immunity by inducing cell death in neoplastic cells. The cytotoxic effector functions of these cells are supported by IFNγ released from T_H1 and type I NKT cells, as well as by self-production of IFNγ that further drives T_H1 polarization. T_H2 polarization opposes T_H1 polarization, and the release of IL-4 and IL-13 by both T_H2 and type II NKT cells can direct macrophages towards an M2 phenotype. Macrophages polarized by IL-4 promote metastasis through the release of EGF, while production of TGFβ suppresses the immune response directly, or indirectly through promotion of T_{Reg} development. In the presence of IL-6, TGFβ can also promote T_H17 polarization. IL-17 induces the production of IL-6 by tumor cells, which both promotes tumor cell growth and further drives T_H17 polarization, while IL-21 has been shown to enhance CTL effector function. Multiple cell types and pathways have been omitted for clarity.

Not surprisingly, $CD4^+$ T cell-deficiency (and elimination of T_H1, T_H2, T_H17 and most T_{Reg} cell populations) has differential effects in different mouse tumor models [19,74–77]. In general, T_H1 polarization is related to anti-tumor effects, while T_H2 polarization is thought to promote tumor formation [20,78,79]. Direct targeting of T_H1 development and effector functions, through IL-12 and IFNγ respectively, clearly indicate a role for T_H1 in tumor rejection [80]. Genetic deficiency in IFNγ or IFNγ receptor 1, or anti-IFNγ antibody treatment, increases MCA-induced sarcomas [81,82], with loss of IFNγ also shown to increase the rate of spontaneous lymphomas and lung adenocarcinomas [83]. Similarly, IL-12 genetic deficiency increases the frequency of chemically induced sarcomas [84] and papillomas [85], while exogenous IL-12 treatment has the opposite effect [86]. Notably, IL-12 dependent rejection of a sarcoma cell was blocked by administration of neutralizing anti-IFNγ antibodies [87]. IFNγ production is not limited to T_H1 cells however, and production by $CD8^+$ T cells, γδ T cells, NK cells, and NKT cells is also dependent upon IL-12 [88]. In one study, loss of IFNγ expression by γδ T cells was found to account for the increase in MCA-induced carcinogenesis in IFNγ-deficient mice [89]. Thus, at least in the skin where a higher percentage of γδ T cells are found, the importance of T_H1 cells may be minimal.

Perhaps the best evidence for a specific role for T_H1 polarization is in mice deficient for signal transducers and activators of transcription protein 6 (STAT6), which display increased T_H1 polarization due to the block in IL-4 signaling [16]. These mice were able to reject tumors formed through injection of a mastocytoma cell line that grew permissively in normal mice [90] and were more resistant to growth of the 4T1 mammary carcinoma cell line [91]. Cells from the lymph node of tumor bearing STAT6-deficient mice produced more IFNγ following secondary stimulation [90], while splenocytes from these mice displayed increased killing against the cell lines [90,91]. STAT1-deficient mice meanwhile display increased T_H2 polarization due to the block in IFNγ signaling, and are more susceptible to tumor development [92]. These results are consistent with the notion that T_H1 polarization increases IFNγ production, leading to more robust anti-tumor immunity through improved CTL responses. Importantly, direct effects of IFNγ on inhibiting proliferation, promoting apoptosis, and inhibiting angiogenesis have been observed, and loss of sensitivity to IFNγ reduces the immunogenicity of tumors [88]. Intriguingly, IFNγ has been found to increase expression of MHCI on MCA-induced sarcomas, thereby improving CTL killing [93], suggesting an addition mechanism by which IFNγ production by T_H1 and other lymphocytes may assist anti-tumor immunity.

By virtue of reduced IFNγ production, T_H2 polarization is likely to be detrimental to the anti-tumor response. T_H2 polarization is dependent upon, and leads to, production of IL-4. This differs from T_H1 and T_H17 polarization, which are not induced by their respective cytokines, although these are involved in lineage stabilization [10,55]. In addition to reducing T_H1 polarization, IL-4 may have direct immunosuppressive effects on $CD8^+$ T cells, as *in vitro* activation of naïve $CD8^+$ T cells in the presence of IL-4 reduces effectiveness of adoptive transfer of tumor-specific transgenic T cells [94,95]. It should be noted however, that these cells, termed T_C2 (as opposed to T_C1 $CD8^+$ T cells activated in the presence of IL-12), were still able to improve survival from B16 melanoma cells in the lung when transferred in greater quantities [95]. Subsequent work by the same group showed that IL-4 and IL-5 expression by the adoptively transferred T_C2 cells was important in mediating this effect [96]. Recombinant expression of IL-4 by several tumor lines also greatly improves clearance [97], and one

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study found that ovalbumin (OVA)-specific T_H2 polarized cells helped clear B16 melanoma lung colonies expressing OVA through recruitment of eosinophils [98]. Is IL-4 production, and by extension T_H2 polarization, therefore protective? Probably not, since the increased anti-tumor response does not depend upon endogenous IL-4, but instead upon an effective T_H1 and CTL response by infiltrating leukocytes [96,99]. By promoting inflammation and leukocyte recruitment, IL-4 production does appear to improve anti-tumor immunity. However, this protective effect is time-dependent, with later stage tumors more resistant to the adoptive transfer of IL-4 producing $CD8^+$ cells [95]. Adoptive transfer of IL-4 producing T_H2 cells, or administration of IL-4 intravenously, also increases lung colonization of B16 melanoma cells injected intravenously [100]. Thus, while IL-4 has the potential to improve anti-tumor immunity, its use may be limited therapeutically to inducing acute inflammation prior to the development of a chronically inflamed tumor microenvironment, with a T_H2 type response itself being detrimental for anti-tumor immunity.

Also produced by T_H2 cells, IL-13 affects the immune response in many of the same ways as IL-4 through activation of Stat6 [101]. As with IL-4, IL-13 expression by tumors can improve the anti-tumor response [102], while endogenous IL-13 can inhibit anti-tumor immunity [103]. T cells do not express the type II IL-4 receptor necessary for binding to IL-13 however, and IL-13 instead appears to inhibit the CTL response indirectly by increasing TGF-β production by myeloid cells in the tumor [104]. Notably however, the source of this IL-13 did not appear to be T_H2 cells in this model, but instead NKT cells [103]. Both IL-4 and IL-13 are considered to be the inducers of the M2 phenotype in monocytes/macrophages, reducing inflammatory cytokine expression and increasing expression of immune suppressive cytokines, resulting in indirect immunosuppression [7,105]. We have recently reported that IL-4 also affects late-stage mammary cancer development by mediating the activity of tumor-associated macrophages (TAMs) [77]. In the MMTV-PyMT mouse model of mammary carcinogenesis, lung metastasis was dramatically reduced by genetic deficiency of either CD4 or IL-4Rα (which would prevent IL-4 and IL-13 signaling). *In vitro*, IL-4 expression by T_H2 cells was found to increase expression of epidermal growth factor (EGF) by TAMs, enhancing the ability of neoplastic cells to extravasate into the circulation. IL-4 and IL-13 may also affect tumor growth independent of their effects on the immune system, as many cell types express the type II IL-4 receptor that binds both IL-4 and IL-13 [106]. This has not been addressed by many studies, but IL-4 has been shown to reduce angiogenesis by inhibiting endothelial cell migration [107,108]. IL-4 and IL-13 also inhibit proliferation of several epithelial cancers, although IL-13 can also promote proliferation and/or inhibit apoptosis of some hematological malignancies [101] as well as breast carcinomas in some models [109].

1.5. Natural killer T cells

NKT cells play a key regulatory role in directing a T_H1 or T_H2 polarized immune response through the rapid production of IFNγ, TNFα, IL-4, and IL-13 following stimulation. This is observed in mice deficient for NKT cells, as infections in these mice, particularly bacterial or parasitic, are often more severe [110]. As with conventional CTL and T_H cells, NKT cells develop in the thymus and express the α and β chains of the TCR. Instead of recognizing a peptide presented by MHC class I or II molecules however, the TCR expressed by NKT cells recognizes glycolipid antigens presented by CD1d, a non-classical member of the MHC family [14]. Type I, or invariant, NKT cells (*i*NKT) express a specific alpha chain variable (V) and joining (J) region (Vα14-Jα18 in mice, Vα24-Jα18 in humans) in combination with a limited number of β chains, and were identified for their ability to recognize α-galactosylceramide (α-GalCer). Type II NKT cells, while also recognizing CD1d, express a variety of αβ TCR chains and are activated by glycolipids that remain poorly defined.

Interest in targeting NKT cells for anti-cancer therapy began with the discovery that treatment with α-GalCer increased the survival time of mice injected with B16 melanoma cells [111]. The anti-tumor effects of α-GalCer [112] and IL-12 treatment [113] were subsequently found to depend upon the presence of *i*NKT cells. Jα18-deficient mice, that lack *i*NKT cells, were also found to be more susceptible to MCA-induced fibrosarcomas [84]. Although capable of directly lysing tumor cells in a perforin-dependent manner, a series of studies by Godfrey and colleagues utilizing MCA-induced fibrosarcomas demonstrated that both NKT-dependent immune surveillance and protection provided by IL-12/α-GalCer therapy was dependent upon IFNγ production by NKT cells, leading to CTL-dependent and NK-dependent anti-tumor responses, respectively [84,114,115]. It has yet to be determined if these same mechanisms are at play in solid tumors of epithelial origin or in models of de novo cancer development.

In addition to possible direct effects of IFNγ production by NKT cells on CTL and NK cells, α-GalCer treatment has been shown to act as an adjuvant through NKT-dependent DC activation and IL-12 production [116,117]. Pulsing DCs *in vitro* with α-GalCer prior to adoptive transfer also more effectively prevents liver metastasis of B16 melanoma cells than injection of α-GalCer [118], possibly by improving long-term IFNγ production and limiting T_H2 cytokine expression [119,120]. Unfortunately, despite these successes in murine tumor transplantation models, injection of α-GalCer, α-GalCer pulsed DCs, or transfer of α-GalCer activated NKT cells all proved ineffectual in early clinical trials in patients with a range of cancer types, even though NKT activation was evident [121].

Using transplantable sarcoma models in which immune surveillance was evident, work by Berzofsky and colleagues found increased resistance of CD1d-deficient mice to tumor development [122]. This was due to the absence of IL-13 producing NKT cells in these mice [103], which were responsible for promoting TGFβ production by splenic $CD11b^+Gr\text{-}1^+$ immature myeloid cells in a model using NIH/3T3-derived cell lines [104]. By comparing Jα18-deficient (lacking type I *i*NKT cells) to CD1d-deficient mice (lacking both type I and type II NKT cells), they concluded that type II NKT cells were responsible for inhibiting immune surveillance in several models where CD25-depletion had no effect [123]. Importantly, in agreement with other studies, α-GalCer treatment increased protection in these models [124]. Meanwhile, stimulation of at least a portion of type II NKT cells with a sulfatide compound reduced protection, and could even counteract the protection offered by α-GalCer treatment [124]. The most parsimonious explanation for these observations is that IFNγ production by type I NKT cells improves the CTL and NK cell responses, while IL-13 production by type II NKT cells inhibits the immune response (Fig. 2). As T, NK, and NKT cells are unresponsive to IL-13, the results also suggest that the effects of NKT cells depend on an intermediate cell, such as DCs [125]. It should be noted however that studies using other tumor injection models found that improved protection of CD1d-deficient mice was not related to IL-13 [126,127]. Protection through adoptive transfer of NKT cells is also dependent on whether the NKT cells are $CD4^+$ or $CD4^-$, and upon the tissue used to isolate NKT cells, which relates at least partially to production of IL-4 [128]. In humans, peripheral blood $CD4^-$ NKT cells expressed only IFNγ and TNFα, whereas $CD4^+$ NKT cells produced both T_H1 and T_H2 type cytokines following stimulation [129,130]. A possible role for cytotoxic effector functions by NKT cells can also not be ruled out. Song and colleagues found that human NKT cells can directly kill monocytes

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pulsed with lysate from human neuroblastoma cell lines [131]. Using immunocompromised mice, the authors found that human NKT cells reduced the number of monocytes in the tumors and inhibited growth of the xenograft, indicating that NKT cells also inhibit pro-tumor immunity by killing tumor-associated macrophages. Tissue localization and tumor type may therefore greatly affect cytokine expression by NKT cells and determine whether they promote or inhibit the anti-tumor response.

2. Conclusions

Although first described over 20 years ago [132], the complexity by which the immune system directs a T_H1 or T_H2 response is only now being appreciated. The concept of polarized populations of immune cells has now been expanded to include $CD8^+$ CTLs, macrophages, and NKT cells. Whether these populations can be defined by genetic programs remains to be determined, but the effects of this polarization on the anti-tumor response demonstrate the importance for further research. Inhibiting immune suppression by blocking the activity of regulatory immune cells, or blocking self-suppression of the CTL response by inhibitory molecules such as PD-1 and CTLA-4, holds great potential for improving anti-tumor responses. These approaches will likely be enhanced by therapies that also dampen the effect of pro-tumor immune molecules released by other leukocytes that enhance angiogenesis, tissue remodeling and cell survival pathways, and in combination, may increase clinical efficacy of adoptive transfer therapies to engender durable anti-tumor immunity. Early clinical results have shown success in some of these areas, although the ability to inhibit peripheral tolerance and improve the anti-tumor response appears directly related to the severity of autoimmunity that is also induced. Being able to release the full potential of the immune response, while also being able to appropriate direct and control that response, is key to the future of immunotherapy.

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Brian Ruffell obtained his Ph.D. from the University of British Columbia in 2008 following his investigations into the role of the cell adhesion molecule CD44 in the immune response. He joined the laboratory of Dr. Lisa Coussens at the University of California, San Francisco shortly thereafter as a postdoctoral fellow to purse his research interests in the role of inflammation in cancer progression and metastasis, with a particular interest in immune suppression within the tumor microenvironment.

David Denardo earned his Ph.D. from Baylor College of Medicine in 2005 where he evaluated mechanisms of non-classical estrogen-induced breast cancer growth. His postdoctoral studies at USCF have focused on the role of adaptive immunity in regulating metastatic breast cancer. His recent studies have elucidated a novel role for T_H2 CD4+ T lymphocytes in potentiating pulmonary metastasis through cross-talk with tumor-associated macrophages, as well as utilizing this realization to develop an immune based signature that predicts breast cancer survival.

Nesrine I. Affara obtained her Ph.D. in Molecular, Cellular, and Developmental Biology from Ohio State University. She joined the laboratory of Dr. Lisa Coussens at the University of California, San Francisco in 2006 where she is currently a postdoctoral fellow. Her current research is focused on identifying molecular mechanisms by which recruitment of innate immune cells into premalignant tissues is regulated by humoral immunity in a transgenic mouse model of epithelial carcinogenesis.

Lisa M. Coussens. Dr. Coussens is a Professor in the Pathology Department and Helen Diller Family Comprehensive Cancer Center at the University of California, San Francisco. The Coussens lab focuses on the role of immune cells and leukocyte proteases as critical regulators of solid tumor development, including skin, lung (non-small cell and mesothelioma) and breast cancer development and is interested in understanding the molecular mechanisms that regulate leukocyte recruitment into neoplastic tissue and the subsequent regulation those leukocytes exert on evolving cancer cells. Dr. Coussens received her Ph.D. in Biological Chemistry from UCLA in 1993, and completed her postdoctoral fellowship in Cancer Biology at the University of California, San Francisco in Douglas Hanahan's laboratory.

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